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PRINCIPAL INVESTIGATOR: Mr. Tao Pan

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, IL 60637

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Mr. Tao Pan

E-Mail: taopan@uchicago.edu

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14. ABSTRACT

Engineered transfer RNAs (tRNA) can be used to reprogram the genetic code and synthesize proteins of interest with new cellular properties. We developed a new class of tRNA mutants that promote numerous and harmful amino acid substitutions upon entering human cells and induce apoptosis. Our objective is to target this tRNA prototype (called killer-tRNA) specifically to cancer cells but sparing healthy blood stem cells and progenitors thereby developing the killer-tRNA as a potential therapeutic agent. Because the killer tRNAs share the exact same feature as all cellular tRNAs, they are undetectable and cannot be cleared by any defense mechanism, thereby constituting a perfect molecular Trojan horse for cell damage and cell death. We aim to test nanocarriers containing killer-tRNAs to human blood cancer cells (acute myeloid leukemia and multiple myeloma) via the high-affinity folate receptors. Since the action of the killer-tRNA is on the synthesis of cellular proteins, it is not restricted to specific stages of cancer. Killer-tRNA can be used to reduce growth of the primary tumor, limit metastatic spread or target tumors at anatomical sites where surgery is difficult to perform.

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tRNA, therapy, delivery, blood cancer.

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Final Report

Proposal: W81XWH-10-0103, Testing Delivery Platforms for New Anticancer tRNA-Based Drugs

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PIs: Tao Pan, Amittha Wickrema

INTRODUCTION

We engineered a transfer RNA chimera that is aminoacylated with amino acid serine but reads the codons for isoleucine. Once inside cells, it drastically increases the amount of misfolded proteins, causing tremendous ER stress leading to massive apoptosis (1). This potent apoptotic inducer, termed killer-tRNA (ktRNA), was designed with the long-term objective to purge healthy stem cells from malignant cells before autologous transplant in multiple myeloma (MM) patients (Fig. 1). The goal of the proposal was to specifically target malignant cells over-expressing folate receptors (FR^+) by linking killer-tRNA to folate and develop adapted tools to facilitate the monitoring of our delivery approach.

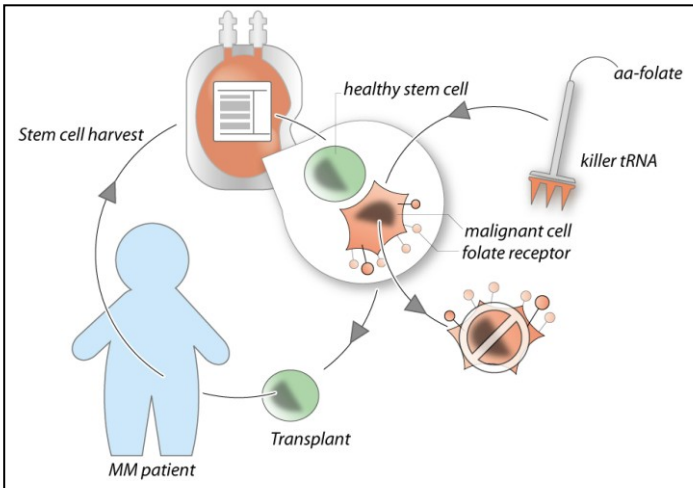

Fig. 1. *Description of the project.* Stem cells harvested from each MM patient will be treated with killer-tRNA to eliminate malignant cells. The remaining healthy stem cells will be transplanted back into the same patient. The goal of the project was to develop a system to deliver killer tRNA only to the malignant cells which have very high expression levels of the folate receptor.

BODY

Proof of concept: killer tRNA triggers the expected cell death response in Myeloma cell lines.

When entering human cells engineered killer tRNA triggers a consistent three steps response within a 3 day span: first translation shuts downs, next cell growth stops and finally cells die massively by apoptosis (1). We tested the sensitivity of two myeloma cell lines, U266 and RPMI to ktRNA. These cells were transfected with ktRNA or wild-type tRNA control together with a plasmid encoding GFP; green fluorescence was used to measure the impact on the translation rate (Fig. 2). As expected, we found the response to ktRNA to be dose dependant. We established that 200 ng of ktRNA (8 pmol) slows down the overall cellular translation rate by a factor 40. We thus confirmed that ktRNA is a broad toxic agent and that its mode of action is not cell type dependant.

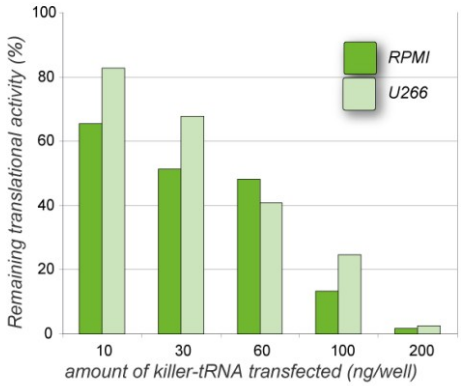

Fig. 2. *Killer tRNA kills multiple myeloma cells.* The graph shows the relative % of translation in cells when treated with killer tRNA. The y-axis is normalized to the same treatment with the wild-type tRNA (100% for each tRNA concentration used).

Task 1: Establish engineered folate receptor positive cancer cell lines (eFR^+) expressing both constitutive (Green Fluorescent Protein) and conditional (Luciferase) reporter. **→Completed successfully.**

Step 1: Design of a plasmid encoding meGFP (constitutive) and mCherry (conditional).
The two reporters have distinct excitation and emission spectrums allowing simultaneous study by flow cytometry and fluorescent microscopy. The gene encoding the fusion protein harbors a non-sense mutation in the linker between meGFP (green) and mCherry (red). In the absence of suppressor tRNA, only meGFP could be expressed (tested here with Hela cells for convenience of transfection). Co-transfection of suppressor tRNA allowed the read-through of the non-sense mutation and supported the expression of the full-length dual reporter (Fig. 3). We thus established the basis for a simple screening procedure to monitor tRNA incorporation into cells based on fluorescence.

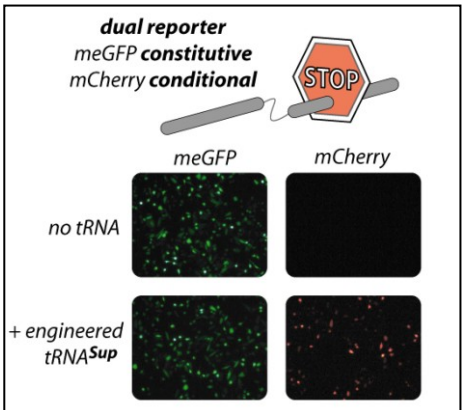

Fig. 3. *Dual reporter works.* The GFP-mCherry dual reporter with a stop codon in mCherry is shown on top. Cells show red fluorescence (from mCherry) only when the engineered suppressor-tRNA is present. Simultaneous measurement of GFP in both panels is shown as controls.

Step 2: selection of FR^+ MM and other cancer cell lines expressing this dual reporter.
Prolonged expression of the reporter was found to be slightly toxic, thus preventing selection of stable myeloma or other cancer cell lines (Myeloma: U266, Myeloid leukemia: KG1, Mantle Cell: JEKO) with the reporter DNA construct integrated into the genome. As an alternative, we found that we can perform 6 hours-transient transfection of FR^+ MM cells with the reporter plasmid prior to testing the incorporation of tRNA.

Task 2: Design tRNA scaffolds that enable the expression of the conditional reporter.
→Completely successfully.

In comparison with standard lipofection used in the above proof of concept studies, efficiency of folate-mediated delivery is expected to be a in the low to medium range. The onset of the apoptotic response associate with ktRNA is dose dependant, so rather than waiting for complex phenotypical changes in the targeted cells we proposed to design a fluorescent screening procedure to monitor the internalization and the usage in translation of killer tRNA based therapy. The concept of ktRNA encompasses a set of three similar molecules, killer-tRNA, wild-type tRNA, and

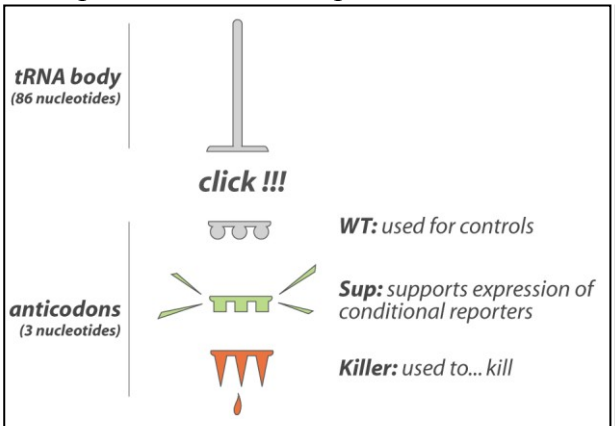

Fig. 4. *The engineered tRNA set for killer tRNA studies.* All three have the same tRNA body (i.e. same sequence outside the anticodon). The anticodon for WT codes for serine, Sup codes for UAG, and Killer codes for isoleucine.

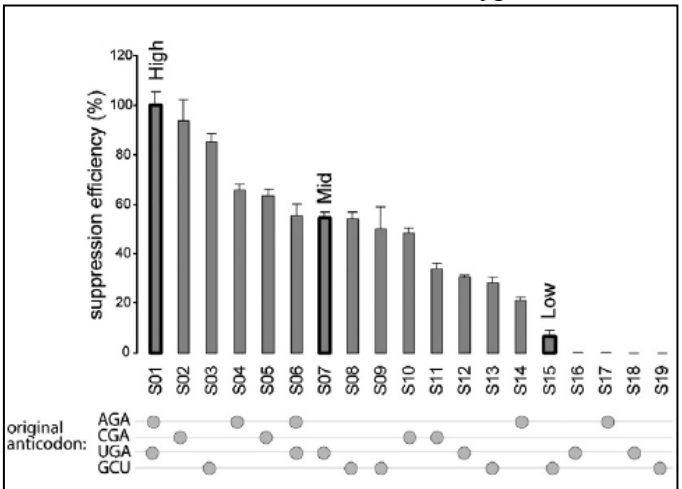

Fig. 5. *tRNA body sequence has a large effect on translational efficiency.* All 19 $tRNA^{Ser}$ sequences are derived from the human genome.

suppressor tRNA. They share identical $tRNA^{Ser}$ bodies (96 % of the molecule) but display different anticodons (Fig. 4). Suppressor tRNA was engineered to base pair with amber stop codons and support translation of reporters harboring amber non-sense mutations in their open reading frame. We tested 19 $tRNA^{Ser}$ scaffolds for their ability to suppress stop mutations and selected the most effective for the subsequent experiments (Fig. 5). Delivery strategies effective for the suppressor tRNA will be directly applicable to its toxic mimic, ktRNA.

Task 3: Condense synthetic tRNA in folate-conjugated nano-particles. Link folate to tRNA and study internalization. **→Continuing, future studies pending.**

We first attempted to package killer tRNA in nanoparticles. We found however, that the efficiency of our nanoparticle packaging method was significantly below the threshold needed to deliver sufficient amount of killer tRNA to kill (data not shown). Our current strategy is to bypass the nanoparticle packaging step entirely by directly attaching folate on the killer tRNA. This strategy should work well for our intended usage of killer tRNA for purging malignant cells in bone marrow stem cells (see Fig. 1). Here, folate-conjugated killer tRNA can be directly added to the harvested stem cells to enable folate receptor mediated delivery.

The presence of a bulky modification like folate can interfere with the function of engineered tRNA and limit its toxic impact on the target cell (2). To keep the integrity of the tRNA body and its ability to interact with the different partners of the translation machinery, we chose to attach folate directly onto the amino acid at the 3' end of the tRNA, away from all interacting motifs needed in translation. Although the ester bond linking the amino acid to the tRNA is known to be labile at alkaline pH, we demonstrate that aminoacylated tRNA used in our study is sufficiently stable in the culture media to allow direct application of cell delivery (Fig. 6).

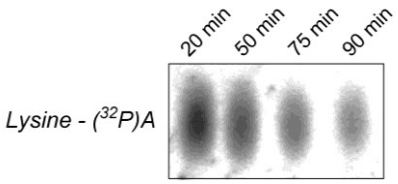

Fig. 6. *Stability measurement of aminoacylated killer tRNA.* Killer tRNA aminoacylated with lysine via a ribozyme is sufficiently stable in the culture media to enable applications of delivery via cell surface receptors.

To obtain high yield of conjugated killer tRNA, we used lysine as the bridging molecule between folate and the 3' end of killer tRNA (Fig. 7). Lysine side chain displays an amine group suitable for covalent attachment of folate. Aminoacylation (transfer of amino acid onto tRNA) is usually performed *in vitro* using purified aminoacyl-tRNA synthetases. Since these enzymes recognize only their cognate substrate, lysine-$tRNA^{Ser}$ cannot be produced by any natural aminoacyl-tRNA synthetase due to substrate incompatibility. We chose instead an RNA enzyme to perform this aminoacylation reaction. The

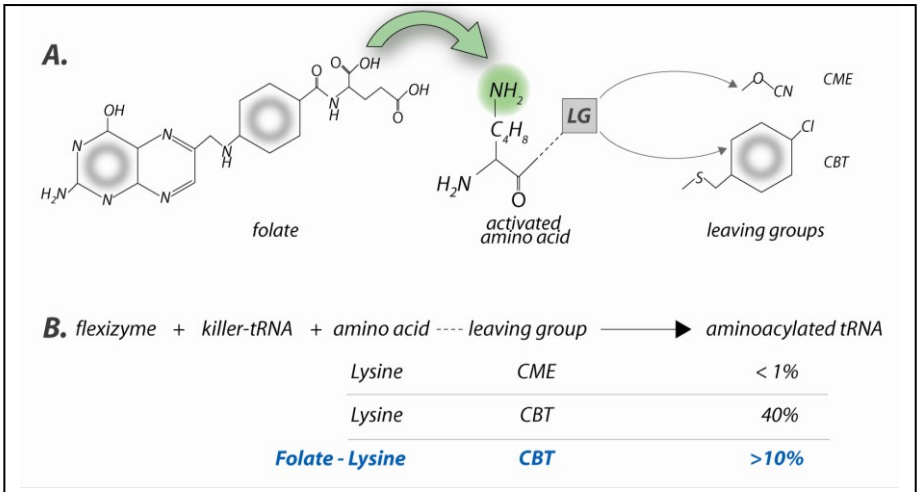

Fig. 7. *Attachment of folate to the 3' end of killer tRNA.* (A) Folate is directly linked to the lysine side chain via chemical synthesis. The carboxyl group of lysine is activated chemically via CME or CBT. (B) Reaction yield of attaching lysine or folate-linked lysine to killer tRNA via flexizyme. The yield of attaching folate-lysine to tRNA is good enough for subsequent cell experiments.

flexizyme is an artificially evolved RNA enzyme designed to aminoacylate tRNA regardless of their specificity (3). The flexizyme is a small RNA that catalyzes the aminoacylation of lysine to any tRNA using a chemically activated amino acid. The flexizyme arranges all the substrate in the right reactive conformation, promotes the formation of the ester bond between the tRNA and the amino acid and the departure of the leaving group. All the substrates necessary for the reaction were produced in our lab either enzymatically (tRNA and flexizyme) or via chemical synthesis (activated amino acid).

We tested two types of activated lysines (Fig. 7). The first (CME) supported very poor aminoacylation to killer tRNA. We were able to increase this yield to 40% by substituting CME for another leaving group CBT. Since March 2011, we were able to optimize the reaction conditions and the type of folate-activated lysine to increase the yield of aminoacylation of tRNA with folate-lysine to over 10% (Fig. 7). This yield is sufficiently high to enable subsequent studies of delivering killer tRNA to folate-receptor over-expressing tumor cells. Unfortunately, additional funds will be needed for these subsequent studies.

Task 4: Study internalization of tRNA via folate receptors into eFR^{+}. **→Future studies pending.**

This task could not be performed because we did not succeed in making sufficient amount of folate-conjugated tRNA as described under task 3.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that killer tRNA also works in blood cancer cells.
- Identified variations in tRNA body sequence for translational efficiency which helps the design of optimal killer tRNAs.
- Determined that the flexi-ribozyme can be used to attach non-serine amino acids to the killer tRNA. This method has been used in our continuing studies to synthesize folate conjugated killer tRNA.
- Obtained folate-lysine charged tRNAs using the flexi-ribozyme strategy.

REPORTABLE OUTCOME

- A dual GFP-mCherry reporter plasmid DNA useful to monitor delivery of tRNA in live cells.
- Synthetic, activated amino acid lysine.
- Synthetic, activated amino acid lysine-folate conjugate.

CONCLUSION

We obtained proof of concept results for killer-tRNA, our proposed anti-cancer drug, in blood cancer cells. We also obtained additional results that form the basis for future studies to deliver killer-tRNA into designated cancer cells. The delivery strategy we are currently testing is different from standard approaches involving nanoparticles and could lead to a more efficient route in delivering tRNA-based drugs. It is possible that the time-consuming step involving optimization of nanocarrier formulations can be avoided owing to the unique features of killer-tRNA and the possibility to link it directly to the delivery molecule (folate in our study).

List of personnel receiving pay from the research effort

1. Tao Pan (PI)
2. Amittha Wickrema (PI)
3. Renaud Geslain (Research Associate, worked on Tasks 1-3)
4. Qing Dai (Research Associate, worked on Task 3)
5. Hui Liu (Research staff, worked on Task 1).

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